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(54) Title: PEPTIDES INCLUDING CTL EPITOPES OF HIV PROTEINS AND USE THEREOF			
(57) Abstract Peptide fragments of HIV proteins such as NEF protein, GAG protein, and ENV protein, which include a CTL epitope. Such peptide fragments containing CTL epitopes may be used to induce or augment cellular immune responses against HIV virus to treat or prevent infection of the animal with HIV virus.			

* See back of page

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**PEPTIDES INCLUDING CTL
EPITOPES OF HIV PROTEINS
AND USE THEREOF**

This invention relates to peptides which include a CTL epitope. The peptides or derivatives thereof may be used to induce and/or augment a CTL response. More particularly, this invention relates to HIV (Human Immunodeficiency Virus) proteins and peptide fragments which include CTL epitopes, and which may be used to induce a CTL response to HIV virus.

Cell-mediated immunity (or CMI) of virus infections is thought to be a major line of host defense against viral infections. CMI may be significant in the development of an effective vaccine against human immunodeficiency virus (HIV), or AIDS virus because HIV vaccines and/or therapies based on the generation of passive transfer of HIV-specific antibody in the absence of cell-mediated immunity have not yielded consistent protection in primates challenged with the HIV virus. Thus, interest has turned to the induction of cell-mediated immune responses to HIV and to the identification of specific epitopes of HIV proteins that stimulate a cytotoxic T lymphocyte, (or CTL) response.

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In accordance with an aspect of the present invention, there is provided a peptide fragment which includes an epitope which is recognized by cytotoxic T lymphocytes (CTL) induced by an HIV protein or CTL epitope thereof. The HIV protein is preferably an HIV-1 protein.

In a preferred aspect, the peptide fragment is recognized by CTL induced by the NEF, GAG, or ENV protein, or a CTL epitope thereof.

The term "peptide fragment" as used herein means a peptide having a number of amino acid residues less than the number of amino acid residues present in the native protein which includes the CTL epitope.

A peptide fragment which includes an epitope which is recognized by CTL induced by an HIV protein is sometimes herein referred to as a peptide fragment containing a CTL epitope.

The peptide fragment may be a fragment of the native protein or an analogue or derivative thereof, provided that such peptide fragment is recognized by CTL induced by the native protein or CTL epitope of such native protein.

The peptide fragment may be comprised of only the amino acid residues which form the CTL epitope, or it may include additional amino acid residues; however, as hereinabove noted, the fragment contains less amino acid residues than the native protein.

In accordance with one embodiment, there is provided a peptide fragment which includes an epitope which is recognized by CTL induced by the NEF protein of HIV-1 (hereinafter sometimes referred to as NEF protein). The peptide fragment which includes a CTL epitope which is recognized by CTL induced by NEF protein may be a fragment of the NEF protein containing such a CTL epitope or an analogue or

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derivative thereof. The CTL epitope of the NEF protein is contained in amino acid residues 73 to 97 of NEF protein according to the Los Alamos, National Laboratory HIV sequence data base as follows (single letter amino acid code)

QVPLRPMT(Y or F, preferably Y)KAAVDLSHFLKEKGGL, and more particularly, such CTL is comprised of residues 73 to 82 (Los Alamos sequence), which have the following structure as defined by the single letter amino acid code:

QVPLRPMT(Y or F, preferably Y)K.

In accordance with another embodiment, there is provided a peptide fragment which includes an epitope which is recognized by CTL induced by the GAG protein of HIV-1 or CTL epitope thereof (sometimes hereinafter referred to as GAG protein). The peptide fragment which includes a CTL epitope which is recognized by CTL induced by GAG protein may be a fragment of the GAG protein containing such a CTL epitope or an analogue or derivative thereof. The CTL epitope of the GAG protein is contained in amino acid residues 179 to 193 of the GAG protein, which has the following structure as defined by the single letter amino acid code:

ATPQDLN(T or M, preferably T)MLN(T or I, preferably T)VGG.

In accordance with yet another embodiment, there is provided a peptide fragment which includes an epitope which is recognized by CTL induced by the ENV protein of HIV-1 or CTL epitope thereof (sometimes hereinafter referred to as ENV protein). The peptide fragment which includes a CTL epitope which is recognized by CTL induced by ENV protein may be a fragment of the ENV protein containing such a CTL epitope or an analogue or derivative thereof. The

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CTL epitope of the ENV protein is contained in amino acid residues 566 to 590 of the ENV protein, which has the following structure as defined by the single letter amino acid code:

LQLTVWGIKQLLAR(I or V, preferably I)LAVERYL(K or R, preferably K)DQ.

In accordance with a further embodiment, there is provided a peptide fragment which includes another epitope which is recognized by CTL induced by the ENV protein of HIV-1 or a CTL epitope thereof. This CTL epitope of the ENV protein is contained in amino acid residues 54 to 80 of the ENV protein, which has the following structure as defined by the letter amino acid code:

CAS (D or E, preferably D)A(K or R, preferably K)(A or S, preferably A)Y(D,S,K, or E, preferably D)(T,K, or P, preferably T)E(V,A,S, or K, preferably V)HN(V or I, preferably V)WA(T or K, preferably T)(H or Q, preferably H)ACVP(T or S, preferably T)(D or N, preferably D)P(N or S, preferably N).

The peptides hereinabove described may be produced by known techniques and obtained in substantially pure form. For example, the peptides may be synthesized on an automatic synthesizer. Journal of the American Chemical Society, Vol. 85, pages 2149-54 (1963). It is also possible to produce such peptides by genetic engineering techniques. The peptide may also be produced by chemical or enzymatic buildup from smaller peptide fragments, or by cleavage of larger peptides or proteins or by solution phase peptide synthesis.

In accordance with another aspect of the present invention, a peptide fragment which includes an epitope, which is recognized by CTL induced by an HIV protein, may be employed in an assay for determining

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HIV infection. More particularly, the assay comprises contacting cells coated with a peptide fragment including a CTL epitope, which is recognized by CTL induced by a HIV protein, with lymphocytes from the animal, and determining lysis of the cells to indicate infection of the animal with HIV. The cells coated with peptide are either autologous cells or an in vitro cell line compatible with the HLA type (MHC allele(s)) of the animal being tested. The peptide fragment containing a CTL epitope may be a peptide fragment of the NEF protein, or a peptide fragment of the GAG protein, or a peptide fragment of the ENV protein or analogs or derivatives thereof as hereinabove described, or mixtures thereof. In general, such an assay is effective for testing an individual of a particular HLA type for CMI responses to said peptide fragments.

In one embodiment, B-cells from the animal are coated with a peptide fragment having a CTL epitope of an HIV protein as hereinabove described. The B-cells may include a detectable label or marker, such as a radioactive chromium marker. The B-cells are then contacted with peripheral blood lymphocytes (or PBL) from the animal. If the peripheral blood lymphocytes contain CTL induced by an HIV protein containing the CTL epitope of the peptide fragment, the CTL will recognize the CTL epitope of the peptide fragment coating the B-cells, and will lyse the B-cells. Lysis of the B-cells will be indicated by release of the detectable marker into the assay medium. If there is lysis of the B-cells as indicated by release of the detectable marker into the medium, then the peripheral blood lymphocytes contain CTL induced by an HIV protein and the animal is infected with HIV.

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In accordance with yet another aspect of the present invention, there is provided a process for augmenting a CTL response which comprises contacting peripheral blood lymphocytes from an animal infected with HIV with a peptide fragment(s) including a CTL epitope as hereinabove described. The peptide fragment preferably contains a CTL epitope of the NEF protein, or a CTL epitope of the GAG protein, or a CTL epitope of the ENV protein. The CTL response may be induced in vitro. The CTL response may be augmented in vitro by incubating peripheral blood mononuclear cells (PBMC's) from seropositive individuals with a peptide of the type hereinabove described.

The peripheral blood lymphocytes having augmented CTL may then be used to treat the animal. For example, PBL augmented in CTL may be administered intravenously as an appropriate physiological suspension (for example in PBS) containing from 10^5 to 10^{10} cells.

In accordance with another aspect, CTL may be induced in vivo by administering to an animal infected with HIV a peptide fragment having a CTL epitope of the type hereinabove described. More particularly, CTL will be induced in vivo by immunization with constructs that contain CTL sites against HIV that have been described above (E.g. Nef7). These vaccines can be formulated using standard techniques with carriers and/or adjuvants (for example, alum, bacterial cell wall components, vegetable oil formulations or derivatives thereof, etc.) to induce CTL responses. In addition, a peptide or peptide derivative having a CTL epitope can be synthesized and/or chemically modified to contain exogenous amino acids and/or fatty acid or

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hydrophobic moieties necessary for induction and/or augmentation of a CTL response. An example of a method of modifying a peptide or peptide derivative having a CTL epitope is described in Deres, et al, Nature, Vol. 342, November 30, 1989, pgs. 561-564. The dosage and administration protocol can be optimized in accordance with standard vaccination practices. A useful dosage for an average adult can be in the range of 1.0 to 1500 micrograms.

The invention will now be described with respect to the following examples; however, the scope of the invention is not to be limited thereby.

EXAMPLE I

(a) Recombinant vaccinia virus construction.

The nef coding sequence was obtained from Dr. Venkatesan, Laboratory of Medical Microbiology, NIAID, NIH, as a cDNA clone. The cDNA was derived from the HIV isolate, NL432 (Adachi et al. (1986) J. Virol. 59, 284). Using standard recombinant DNA technique, the nef gene was excised and placed into a vaccinia virus integration vector. The integration vector contains a vaccinia virus promoter termed P 7.5. Promoter P 7.5 allows expression of foreign genes at early and late times after infection. The nef coding sequence was placed downstream of p 7.5. The P 7.5 nef cassette was inserted into the thymidine kinase (TK) locus of vaccinia virus by standard procedures resulting in insertional inactivation of TK. A TK-recombinant vaccinia virus, designated vTFnef, was isolated and purified. Expression studies were performed with vTFnef to confirm proper expression of Nef. Cell monolayers were infected with vTFnef in the presence of radiolabeled amino acids or myristic acid. Cell lysates were

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immunoprecipitated with Nef anti-serum. A specific 27 kd protein was precipitated. The 27 kd protein corresponds to the predicted molecular size as well as previously reported values for Nef (J.S. Allan et al., Science (1985) 230, 810; G. Franchini et al., Virology (1986) 155, 593). Nef is also myristoylated demonstrating authentic expression of the nef coding sequence.

(b) Identification of individuals with Nef-specific CTL responses. Recombinant virus, vTFnef, was used to screen circulating T lymphocytes for CTL activity directed to Nef from a group of 12 healthy, HIV-1 seropositive HIV-1 patients. Fresh peripheral blood mononuclear cells (PBMC) were obtained from these patients and tested for cytolytic activity against autologous cells infected with vTFnef. Unfractionated PBMC were separated over Hypaque/Ficoll, resuspended in RPMI-1640 medium (M.A. Bioproducts, Walkersville, MD) with 10% fetal calf serum, and assayed on the day of separation as described previously (Koenig et al. (1988) Proc. Natl. Acad. Sci USA 85, 8638-8642). Autologous cells were prepared from B lymphocytes obtained from the same patient tested for CTL activity, i.e. to match HLA types for both target and effector cells. Autologous B lymphoblastoid cell lines from these HIV seropositive patients were prepared by transformation with 'Epstein-Barr Virus (EBV) as described previously (Blumberg et al. (1987) J. Infect. Dis. 155, 877; Koenig et al. (1988) Proc. Natl. Acad. Sci USA 85, 8638-8642). These cell lines were used as targets in an autologous chromium-release assay as described by Koenig, et al. (ibid). In this assay, lysis of labeled target cells results in the release of chromium into the medium. Freshly isolated

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autologous PBMC were used as effector cells in the assay. Two subjects had a Nef-specific cytotoxic response that was approximately five-fold higher than that observed for control vaccinia virus infected target cells.

(c) Mapping a Nef-specific CTL epitope. The Nef-specific CTL response of these two donors was further examined by testing a set of peptides individually that spanned the entire Nef protein sequence. Sixteen peptides covering the nef region were synthesized in overlapping linear sequences, 25 residues in length with 13 residue overlaps. The peptides were produced as described in part (d) below. Autologous cells were pulsed with 25 to 50 μ M peptide for 12 hours before labeling with Cr-51. Chromium-release assays were performed as described (Koenig et al., op. cit.). One peptide was recognized as a CTL target. This peptide, designated Nef7, corresponds to Nef amino acid residues 73 to 97 according to the Los Alamos National Laboratory HIV sequence database. The N- and C-terminal boundaries of this epitope were fine-mapped by peptide deletions (A summary of peptides used for fine-mapping is shown in Table 1). A 10 residue peptide (10-mer), Nef7B, corresponding to residues 73 to 82 (QVPLRPMTYK), was capable of serving as the CTL epitope with the same reactivity as Nef7 (25-mer).

Individuals tested exhibiting Nef CTL activity are designated #1 and #2. Peptide Nef7B is contained within a highly conserved region of the Nef protein sequence (at least 90 percent conserved in all HIV-1 isolates sequenced to date). Although these subjects were classified as seropositive by standard screening assays; detection of antibody responses to Nef was negative by radioimmunoassay.

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(d) Peptide synthesis. Synthetic peptides were constructed using the stepwise solid phase approach of Merrifield (J. Am. Chem. Soc. (1963) 85, 2149) on an Applied Biosystems Model 430A peptide synthesizer. All synthetic peptides were assembled on an insoluble copolymer "PAM" resin consisting of styrene and divinylbenzene.

The symmetric anhydride derivatives were used as the acylating species for all amino acids except asparagine, glutamine, arginine and histidine. These four amino acids were coupled as the 1-hydroxybenzotriazole esters. The reactive side chains of amino acids were protected during the synthesis. The protecting groups used were O-benzyl for Asp and Glu; benzyl for Ser and Thr; p-methylbenzyl group for Cys; tosyl for Arg; dinitrophenyl for His; 2-chlorobenzyloxycarbonyl for Lys; 2-bromobenzyloxycarbonyl for Tyr; and formyl for Trp.

Following their syntheses, peptides were cleaved with anhydrous liquid HF in the presence of anisole, dimethylsulfide and ethanedithiol. Once cleaved the peptides were precipitated in ether, and then extracted from the resin with 30% (v/v) glacial acetic acid in water.

The cleaved peptides from the resin were analyzed and subsequently purified to greater than 95% homogeneity, using a Vydac C-4 reverse-phase column, and Beckman "System Gold" HPLC. The correct amino acid content of each peptide was verified by hydrolyzing purified material with constant boiling in 6N HCl at 150 degrees centigrade for 2 hours. Once hydrolyzed, these samples were then subject to amino acid compositional analysis using the Beckman "System Gold" amino acid analyzer.

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(e) CTL assay. Preparation of PBMCs, generation of autologous lymphoblastoid cell lines and cytotoxicity microassays (CTL) were done as described in part (b) above except that the autologous B lymphoblastoid cell line was coated with the Nef7 peptide instead of being infected with the noted viral vector.

(f) Augmenting CTL production in vitro. A protocol which was used for boosting (augmenting) CTLs in vitro is described as follows. Fresh PBMCs from seropositive individuals were separated over Hypaque/Ficoll, resuspended in RPMI-1640 medium containing 10% human serum and 10 ug/ml of Nef7 peptide and incubated for 3 days. After 3 days, cultures were supplemented with recombinant IL-2 at 5 units/ml. At day 5, cultures were restimulated with fresh PMBC pulsed with Nef7 peptide for 3 hours, irradiated with 3000 rads, and washed to remove excess peptide. Cells were restimulated as before on day 14.

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Table 1

<u>Overlapping Peptides</u>	<u>Positions</u>
QEEEEVGFPVTPQVPLRPMTYKAAV	(61-85)
QVPLRPMTYKAAVDLSHFLKEKGGL	(73-97)
FPVTPQVPLRPMTYKAAVDLS	(68-88)
QVPLRPMTYKAAVDLS	(73-88)
QVPLRPMTYK	(73-82)
VPLRPMTYK	(74-82)
PLRPMTYK	(75-82)
LRPMTYK	(76-82)
QVPLRPMTY	(73-81)
QVPLRPMT	(73-80)
QVPLRPMTFK	(73-82)
PMTYKAAVDLSHFLKEKGGL	(78-97)
AAVDLSHFLKEKGGL	(83-97)
SHFLKEKGGL	(88-97)

The term "animal" as used herein includes humans and non-humans and is preferably a human. It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described, and within the scope of the accompanying claims, numerous modifications may be made of the specific teachings contained herein.

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WHAT IS CLAIMED IS

1. A peptide fragment which includes an epitope which is recognized by CTL induced by one of NEF protein, GAG protein, or ENV protein, or a CTL epitope of such proteins.

2. The peptide fragment of Claim 1 wherein the peptide fragment includes an epitope which is recognized by CTL induced by NEF protein or a CTL epitope thereof.

3. The peptide fragment of Claim 2 wherein the peptide fragment includes a CTL epitope of NEF protein.

4. The peptide fragment of Claim 3 wherein the peptide fragment is comprised of amino acid residues 73 to 97 of NEF protein.

5. The peptide fragment of Claim 3 wherein the peptide fragment is comprised of amino acid residues 73 to 82 of NEF protein, as defined by the single letter amino acid code:

QVPLRPMT(Y or F)K.

6. The peptide fragment of Claim 5 wherein the peptide fragment has the following structural formula:

QVPLRPMTYK.

7. The peptide fragment of Claim 1 wherein the peptide fragment includes an epitope which is recognized by CTL induced by GAG protein or a CTL epitope thereof.

8. The peptide fragment of Claim 7 wherein the peptide fragment includes a CTL epitope of GAG protein.

9. The peptide fragment of Claim 8 wherein the peptide fragment is comprised of amino acid residues

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179 to 193 of GAG protein, as defined by the single letter amino acid code:

ATPQDLN(Tor M)MLN(T or I)VGG.

10. The peptide fragment of Claim 9 wherein the peptide fragment has the following structural formula:

ATPQDLNTMLNTVGG

11. The peptide fragment of Claim 1 wherein the peptide fragment includes an epitope which is recognized by CTL induced by ENV protein or a CTL epitope thereof.

12. The peptide fragment of Claim 11 wherein the peptide fragment includes a CTL epitope of ENV protein.

13. The peptide fragment of Claim 12 wherein the peptide fragment is comprised of amino acid residues 566 to 590 of ENV protein, as defined by the single letter amino acid code:

LQLTVWGIKQLLAR(I or V)LAVERYL(K or R)DQ.

14. The peptide fragment of Claim 13 wherein the peptide fragment has the following structural formula:

LQLTVWGIKQLLARILAVERYLKDQ.

15. The peptide fragment of Claim 12 wherein the peptide fragment is comprised of amino acid residues 54 to 80 of ENV protein, as defined by the single letter amino acid code:

CAS(D or E)A(K or R)(A or S)Y(D,S,K, or E)(T,K, or P)E(V,A,S, or K)HN (V or I)WA(T or K)(H or Q)ACVP(T or S)(D or N)P(N or S).

16. The peptide fragment of Claim 15 wherein the peptide fragment has the following structural formula:

CASDAKAYDTEVHNWATHACVPTDPN.

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17. An assay for determining infection of an animal with HIV virus, comprising:

contacting cells from the animal coated with the peptide fragment of Claim 1 with peripheral blood lymphocytes from said animal, said cells being autologous cells or cells compatible with the HLA type of the animal being tested; and

determining lysis of said cells to indicate infection of said animal with HIV virus.

18. An assay for determining infection of an animal with HIV virus, comprising:

contacting cells coated with the peptide fragment of Claim 4 with peripheral blood lymphocytes from said animal, said cells being autologous cells or cells compatible with the HLA type of the animal being tested; and

determining lysis of said cells to indicate infection of said animal with HIV virus.

19. An assay for determining infection of an animal with HIV virus, comprising:

contacting cells from the animal coated with the peptide fragment of Claim 5 with peripheral blood lymphocytes from said animal, said cells being autologous cells or cells compatible with the HLA type of the animal being tested; and

determining lysis of said cells to indicate infection of said animal with HIV virus.

20. A process for augmenting a CTL response, comprising:

contacting peripheral blood lymphocytes from an animal infected with HIV with the peptide fragment of Claim 1.

21. A process for augmenting a CTL response, comprising:

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contacting peripheral blood lymphocytes from an animal infected with HIV with the peptide fragment of Claim 4.

22. A process for augmenting a CTL response, comprising:

contacting peripheral blood lymphocytes from an animal infected with HIV with the peptide fragment of Claim 5.

23. The process of Claim 20 wherein said CTL response is augmented by systemically administering said peptide fragment to an animal infected with HIV.

24. A process for inducing a CTL response, comprising:

administering to an animal infected with HIV the peptide fragment of Claim 1.

25. A process for inducing a CTL response, comprising:

administering to an animal infected with HIV the peptide fragment of Claim 4.

26. A process for inducing a CTL response, comprising:

administering to an animal infected with HIV the peptide fragment of Claim 5.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US90/05343

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):A61K 39/21; C07K 13/00; C07K 7/04

U.S. CL.: 530/300, 328; 435/4; 424/89

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	530/300, 328; 435/4; 424/89

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Databases: Dialog (Files 157,72,73,399,155,154,5)
Automated Patent System (File USPAT, 1971-1990)

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁸

Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
T	Journal of Immunology, volume 145, No. 1, issued 01 July 1990, Koenig et al., "Mapping The Fine Specificity Of A Cytolytic T Cell Response To HIV-1 <u>nef</u> Protein", pages 129-135.	1-26
X	European Journal of Immunology, Volume 19, issued 1989, Culmann et al., "An antigenic peptide of the HIV-1 NEF Protein recognized by cytotoxic T lymphocytes of seropositive individuals in association with different HLA-B molecules", pages 2383-2386, see entire document.	1-26
X,P	The Journal of Experimental Medicine, Volume 170, issued December 1989, Takahashi et al., "Structure Requirements for class I MHC Molecule-mediated antigen presentation and cytotoxic T cell recognition of an immunodominant determinant of the human immunodeficiency virus envelope protein", pages 2023-2035, especially pages 2023 & 2026.	1-26

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

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"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

06 February 1991

Date of Mailing of this International Search Report

22 FEB 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Lynette F. Smith
Lynette F. Smith ebw

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING¹

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-6, 17-22 to peptide fragments and use thereof
- II. Claims 7-10 to GAG peptide fragment
- III. Claims 11-16 to envelope protein peptide fragments.
- IV. Claims 23-26 to in vivo administration of NEF protein

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

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